ORIGINAL ARTICLE

Elucidation of the Mechanism of Bone Regeneration When a Mixture of β-TCP and Autologous Bone Granules are Transplanted

Masayasu TATSUMURA[1], Kenichirou YASUI[1], Yoshiya HASHIMOTO[2], and Naoyuki MATSUMOTO[1]

[1]Department of Orthodontics, Osaka Dental University, Osaka, Japan
[2]Department of Biomaterials, Osaka Dental University, Osaka, Japan

Synopsis
We examined the amount and maturity of collagen as a parameter for bone quality in the graft material transplanted into a rat skull bone defect model. Male SD rats were used to generate an experimental animal model of skull defects. The obtained autologous bone granules and β-TCP granules were mixed at a ratio of 1:1 to prepare a graft material that combines autologous bone with β-TCP granules. At 8 weeks after transplantation, the β-TCP+ autologous bone and the autologous bone groups showed BV/TV values, measured based on CT image analysis, significantly different from those in the β-TCP and control groups. The β-TCP+ autologous bone group exhibited the ability to form blood vessels into the graft material for a longer period of time after transplantation compared to that in the autologous bone group, as well as the elevated production of collagen into β-TCP granules during the bone formation process compared to that in the β-TCP group.

Key words: *autologous bone graft, β-TCP granules, micro-CT, picrosirius red staining, collagen, vWF immunostaining*

Introduction

In recent years, research has focused on introducing bone regenerative medicine in the field of dentistry. Currently, autologous bone and tricalcium phosphate (TCP) are widely used as bone regeneration materials in clinical settings [1, 2]. Autologous bone grafts, in particular, contain bone-forming cells, such as osteoblasts, and the deposition of inorganic components in collagen secreted by osteoblasts is considered to progress calcification [3, 4]. In addition, with its vascular induction ability, growth factors such as BMP, and osteogenic ability, autologous bone has long been the predominantly used bone regeneration material because its mechanical properties are similar to those of the original bone tissue [1, 5]. However, autologous bone grafting has some drawbacks, such as invasiveness at the time of bone collection and the limited availability of collectable bone. Therefore, the range of its application is limited, and the research and development of alternative artificial graft materials are being conducted [3].

Meanwhile, TCP has been used as an artificial transplant material in clinical settings for many years and is commercialized under various trade names [5, 6]. TCP, which has the chemical formula $Ca_3(PO_4)_2$, has different crystal polygons with a unique firing temperature, specifically α-TCP in the high-temperature stable phase and β-TCP in the low-temperature stable phase. Moreover, these TCP crystal polygons show different properties. Compared with β-TCP, α-TCP is more water-soluble and is replaced by new bone more quickly. However, it becomes susceptible to erosion with tissue fluid and easily disintegrates over time after transplantation [7]. In contrast, although the replacement of β-TCP

with new bone is slow due to its lower water solubility than α-TCP, β-TCP tends to remain in the living body as a scaffolding material even after transplantation. In addition, since both α-TCP and β-TCP can be artificially synthesized, they possess a porous structure that is not found in autologous bone [8]. However, TCP has a lower regenerative bone mass than autologous bone grafts due to its lack of growth factors. Thus, unfortunately, no artificial bone made of a single artificial material with the same or better capacity than autologous bone has been developed.

Bone strength, as well as regenerative bone mass, is considered crucial for the introduction of bone regenerative medicine to the field of dentistry, in which load, such as occlusal force, is applied. Bone strength is composed of two parameters, bone density and bone quality [9]. Whereas bone density indicates the amount of minerals per unit area and volume of bone, bone quality is defined as "the overall characteristics of bone that affect resistance to bone fractures." Thus, an evaluation of bone quality, in addition to bone density, is important in determining bone strength [10]. However, although there have been many reports on bone density, few have studied bone quality. According to the National Institutes of Health, the components of bone quality are subdivided into "bone structure," "bone turnover," "calcification," and "damage accumulation." In recent years, research has focused on bone cells, the orientation of biological apatite crystals, and the bone structure composed of collagen, and it was reported that the properties of collagen can be used as a clinical parameter to evaluate bone quality [11, 12]. In addition to glycoproteins, such as sialoprotein and osteonectin, bone mainly contains type I collagen. Moreover, it has been reported that collagen is closely related to bone remodeling [4, 13, 14].

Focusing on collagen as a parameter to evaluate bone quality, we examined the maturity of collagen in the graft material. Further, bone regeneration is known to require blood supply and circulation through newly formed blood vessels. Based on these points, we aimed to elucidate the mechanism underlying the bone regeneration process by evaluating collagen and angiogenesis in new bone using a regeneration material that combines autologous bone and β-TCP granules.

Material and Methods

Preparation of graft material

The cortical bone, obtained upon creating a defect 9 mm in diameter in the rat calvaria using a trephine bur, was used as autologous bone granules and crushed into particles with a bone mill. The initial particle size of β-TCP granules (Taihei Chemical Industrial Co. Ltd., Osaka, Japan) was 1700 μm at the maximum. Past studies have shown that angiogenesis and bone formation occur in the space between β-TCP granules upon the implantation of β-TCP granules at the bone defect, and granule sizes suitable for maintaining such intergranular spaces have been reported [15]. In this study, the granule size of β-TCP was set to 100–500 μm, which is suitable for maintaining the granular space according to the study [15]. Thus, using sieves with a diameter of 500 μm and 100 μm, the granule size was adjusted to a uniform size of 100–500 μm, which is thought to be the optimal range of granule sizes for graft material. The obtained autologous bone granules and β-TCP granules were mixed at a ratio of 1:1 to prepare a graft material (0.5 g) that combines autologous bone with β-TCP granules.

SEM (field-emission scanning electron microscopy)

The outer surface conditions of the graft material were analyzed using a field emission-scanning electron microscope (FE-SEM; S-4800, Hitachi High-Technologies Corporation, Tokyo, Japan). Prior to observation, the samples were coated with osmium with an HPC-20 osmium coater (Vacuum Device Ltd., Ibaraki, Japan).

Surgical procedure

Forty-eight male Sprague-Dawley rats (age, 8 weeks; body weight, 300–350 g; Shimizu Laboratory Supplies Co., Ltd., Kyoto, Japan) were used in the experiment. A rat anesthetic (10 mL) was prepared by mixing Domitor (0.75 mL), midazolam (2.0 mL), butorphanol (2.50 mL), and distilled water for injection (4.75 mL), and general anesthesia was performed by intraperitoneally administering 0.2 mL of the anesthetic per 100 g of rat body weight. Subsequently, the surgical areas were shaved and the scalp was incised. After the periosteum of the skull was removed to form a flap, the surgical wound was widened to secure the surgical field. A critical-sized bone defect (diameter, 9.0 mm; depth, 1.0 mm) was created at the center of the calvaria using a trephine bur under running water.

Experimental materials were transplanted into the skull defect to prepare three experimental groups as follows: the autologous bone group, the β-TCP + autologous bone group, and the β-TCP group. In addition, animals with the skull defect without any transplantation were used as the control group. Following transplantation, suturing was performed with 2-0 absorbent sutures. At the end of the observation period (4, 6, and 8 weeks after implantation), animals were euthanized via inhalation of an overdose of isoflurane. Animal experiments were approved by the Animal Research Committee of Osaka Dental University (approval number: 19-02006).

CT image analysis

Observations of bone regeneration in the skull defect were performed with the microfocus X-ray CT system (inspeXio, SMX-225CT, SHIMADZU, Kyoto, Japan). After microfocus X-ray CT imaging, a 3D image was created using Ratoc System Engineering.

Analysis of bone volume ratio

After imaging with micro-CT, structural analysis was performed, and the volumes of the bone defect region and the new bone region were measured using the bone morphological measurement program. Moreover, in the bone density measurement using the TRI/3D-BON 3D analysis routine, the bone mineral content of the graft material was measured based on the pre-adjusted HA phantom image. Bone mineral content, expressed as bone mineral density (BMD), is a widely used measure to assess bone health. Use the X ray CT image to determine the BMD value of the new bone. The Ratoc system displays the BMD value in a pseudo-color 3D image and observes the distribution of bone density in each part. Bone defect volume (TV, cm^3) and new bone volume (BV, cm^3) were measured directly, and the bone volume ratio was calculated as BV/TV (%). Based on these values, the mass of hard tissue formed in the defect was evaluated.

Histological evaluation

After observing the presence or absence of gross abnormalities in each organ and tissue in all cases, the organs and tissues surrounding the graft material (including the skull) were extracted and stored in 10% neutral buffered formalin fixative solution. After fixation, the organs and tissues (including the skull) surrounding the graft material were acid-decalcified with EDTA solution. After preparing a paraffin block, the skull grafted area was cut based on the sagittal plane. After confirming the section with the exposed edge of the graft material, microscopic examinations of hematoxylin and eosin (HE) staining and von Willebrand factor (vWF, factor VIII-related antigen) immunostaining, as well as observation of the color of picrosirius-stained specimens under a polarizing microscope, were performed. vWF-positive vascular endothelial cells were stained brown with vWF immunostaining, which was detected to determine whether angiogenesis had occurred [16]. In addition, picrosirius red staining consists of yellow picric acid dye, which is a small molecule, and Sirius Red, which is a polymer. As it binds to a metal complex salt dye, picrosirius red staining has become the basis of the protocol used to stain collagen fibers and related tissues and is also often used to evaluate new bone [17, 18]. In this study, the maturity of collagen fibers was evaluated by considering yellow, orange, and red collagen fibers to be mature collagen and green-like collagen fibers as immature collagen.

Statistical processing

Statistical processing of the obtained BV/TV values (%) was performed. Statistical analysis was performed based on an analysis of variance using the Tukey–Kramer test. Differences with a *p*-value less than 0.05 were considered significant.

Results

SEM

We examined β-TCP + autologous bone granules (Fig. 1a, b, c), autologous bone granules (Fig. 1d, e), and β-TCP granules before and after granulation (Fig. 1f, g and 1h, i, respectively) under an electron microscope. After granulation, while the space between β-TCP granules became smaller, there was no change in the porosity of the β-TCP granules. The particles of autologous bone were larger than β-TCP granules, and the fibrous structure was found on the surface of autologous bone granules. Porous β-TCP granules, as well as autologous bone particles with a fibrous surface, were observed in β-TCP + autologous bone granules, confirming that granulation does not affect the porosity of β-TCP granules.

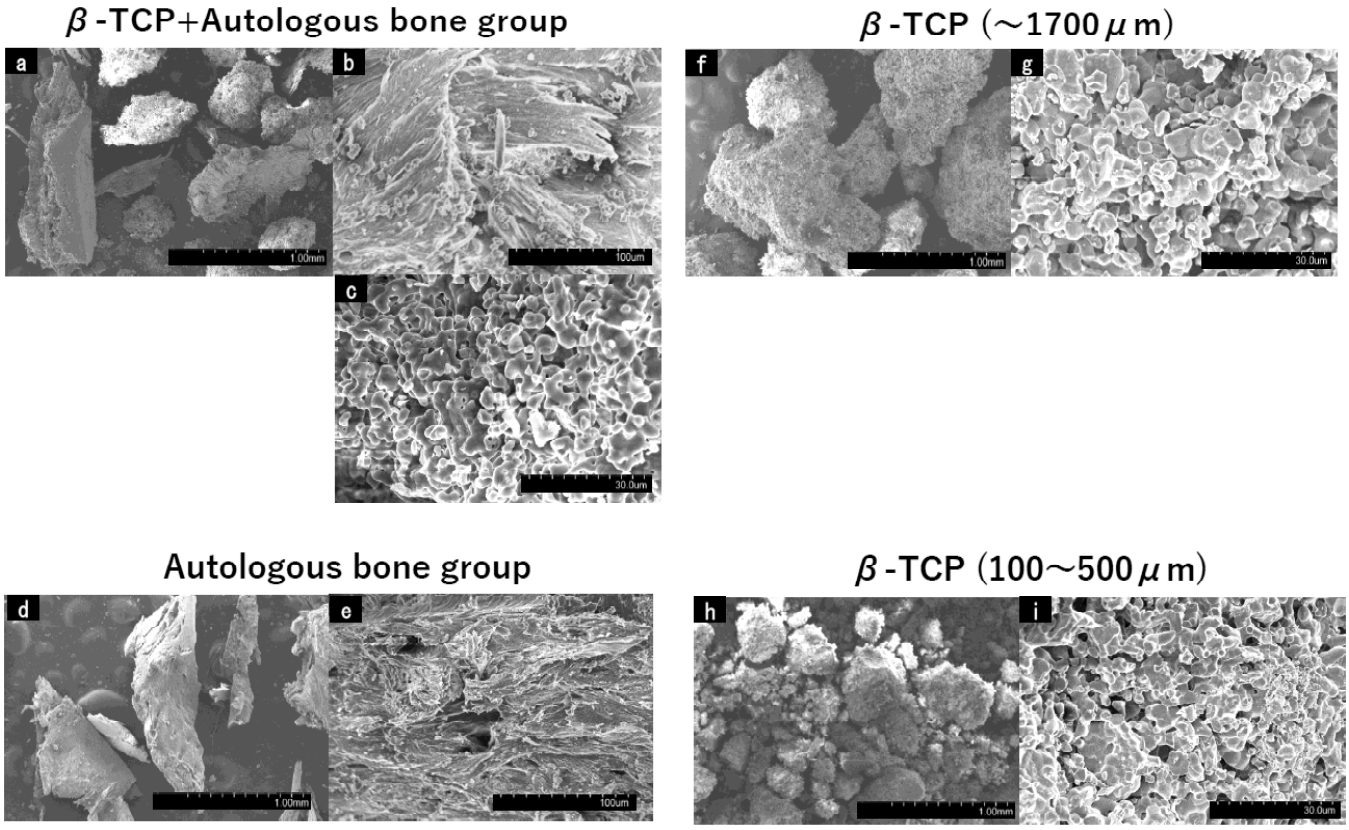

Fig. 1 Scanning electron microscopy (SEM) image (left: low magnification, right: high magnification). (a) Low magnification image of β-TCP + autologous bone granules. (b) Strongly magnified image of autologous bone granules in β-TCP + autologous bone granules; (c) strongly magnified image of β-TCP granules in β-TCP + autologous bone granules. (d,e) Autologous bone granules. (f,g) β-TCP granules before granulation. (h,i) β-TCP granules after granulation. The bars are as follows: a, c, e, g, 1 mm; f, h, 100 μm; b, d, i, 30 μm.

3D color-mapping (3D map) and micro-CT examination

Fig. 2 shows the 3D-reconstructed micro-CT images and bone mineral density (BMD) distribution maps. Each experimental group was analyzed at three time-points of 4, 6, and 8 weeks after transplantation, and the BMD at each time-point is shown in the 3D map. The color scale of BMD is expressed as red and orange for high BMD values, yellow and green for medium BMD values, and light blue and purple for low BMD values.

At 4 weeks after transplantation, medium BMD values (yellow and green) were observed in the autologous bone group. Medium-to-high BMD values (red, yellow, and green) were observed in the β-TCP + autologous bone and β-TCP groups. In the defect group (control group), medium BMD values (yellow and green) were partially observed around the host bone. The micro-CT images showed that the bone defects of the autologous bone, β-TCP + autologous bone, and β-TCP groups were all filled with hard tissue. In contrast, the micro-CT images of the defect group showed that although hard tissue partially formed around the existing bone, it did not completely fill the center of the defect.

At 6 weeks after transplantation, medium-to-high BMD values (red, yellow, and green) were observed in the β-TCP group, and medium BMD values (yellow and green) were observed in the autologous bone, β-TCP + autologous bone, and defect groups. The micro-CT images showed that the bone defects were filled with a small amount of hard tissue. Although hard tissue in the bone defect was seen in all groups, an evident increase in hard tissue from 4 weeks was observed only in the defect group. The micro-CT images showed that the

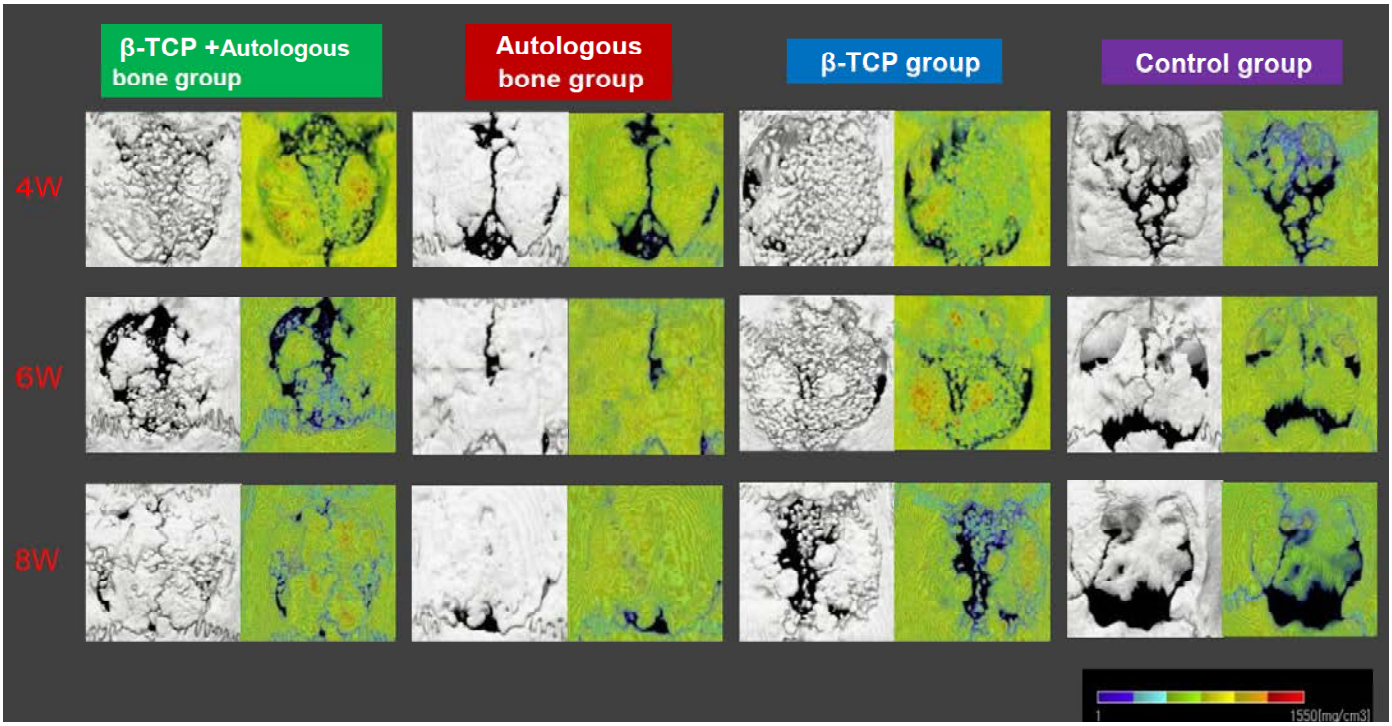

Fig. 2 Micro-computed tomography images and the three-dimensional bone mineral density distribution map at 4, 6, and 8 weeks after transplantation. Blue indicates low bone density, and red indicates high bone density.

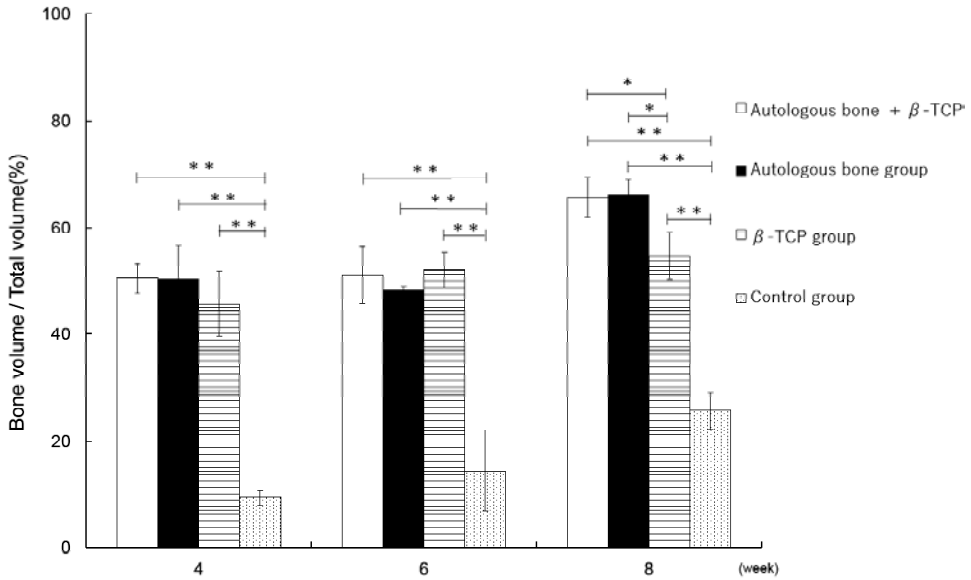

Fig. 3 Analysis of bone volume to total volume ratio (BV/TV; %) in each group to evaluate the quantity of the new bone at 4, 6, and 8 weeks after transplantation (p<0.05).

defect was filled with hard tissue, and the amount of hard tissue in this group increased over time. At 8 weeks after transplantation, the micro-CT images of the defect group showed little changes in the BMD values from 6 weeks. High BMD values were essentially observed in the autologous bone and β-TCP + autologous bone groups, but the β-TCP alone transplanted group showed a slight decrease in the BMD values from 6 weeks.

BV/TV analysis

For the determination of new bone tissue using image analysis software (Ratoc system), the average volume ratio of new bone was calculated by dividing the bone volume (BV) by the total volume (TV). The BV/TV values of new bone relative to the total amount of bone loss at 4 weeks were 50.6% in β-TCP + autologous bone group, 50.4% in autologous bone group, 45.7% in β-TCP group, and 9.3% in control group. The BV/TV values at 6 weeks were 51.2% in β-TCP + autologous bone group, 48.5% in autologous bone group, 52.1% in in β-TCP group, and 14.5% in control group. The BV/TV values at 8 weeks were 65.1% in β-TCP + autologous bone group, 66.1% in autologous bone group, 54.8% in β-TCP group, and 25.7% in control group (Fig. 3).

Fig. 3 shows the BV/TV values of all groups at each time-point. At 4 weeks after transplantation, all transplanted groups had BV/TV values significantly different from those of the control group, but no significant difference in BV/TV values was observed between the transplanted groups. The same trend in results as that at 4 weeks was observed at 6 weeks. At 8 weeks, the BV/TV values of the β-TCP + autologous bone and the autologous bone groups were significantly different from those of the β-TCP and the control groups. However, there was no significant difference in the BV/TV values between the β-TCP + autologous bone and the autologous bone groups.

Histological evaluation

Four-week postoperative groups

HE staining did not reveal any inflammatory cells in the β-TCP + autologous bone, autologous bone, β-TCP, and control groups (Fig. 4). Some autologous bone granules remained in the β-TCP + autologous bone and autologous bone groups, although their replacement with new bone was observed (Fig. 4d-1,2). In the β-TCP + autologous bone group, partial replacement with new bone was observed between the transplanted β-TCP granules, but more than half of the β-TCP granules remained (Fig. 4a-1,2). In addition, the β-TCP group exhibited connective tissue around the β-TCP granules as a whole, and the slight formation of new bone from the direction of the side of the skull (sutured side) was observed. However, replacement with new bone in the β-TCP group was not as advanced as that in the β-TCP + autologous bone group, and most of the transplanted β-TCP granules remained (Fig. 4g-1,2). In the control group, thin new bone was partially observed in the defect. However, it did not form a continuous structure as it was formed by fibrous connective tissue with the host bone, and the replacement with new bone was scant (Fig. 4j-1,2).

In vWF immunostaining, β-TCP + autologous bone group contained a small number of vascular endothelial cells between autologous bone granules, but many vascular endothelial cells were not between autologous bone granules, but of β-TCP granules. It was located in between (Fig. 4a-3). A small number of vascular endothelial cells were found in the autologous bone group (Fig. 4d-3). The β-TCP group clearly showed a more positive response showing vascular endothelial cells identified within the same region compared to autologous bone and β-TCP + autologous bone group (Fig. 4g-3). In the control group, a small number of vascular endothelial cells were observed in the fibrous connective tissue (Fig. 4j-3).

Under a polarizing microscope, picrosirius staining revealed immature collagen around the transplanted autologous bone granules in the β-TCP + autologous bone group, and mature collagen in the β-TCP granules (Fig. 5a). Immature collagen was shown in the new bone matrix coloring of the autologous bone group, and mature collagen such as red was partially observed. Regarding the proportion of collagen types, immature collagen was predominant, but mature collagen was partially present (Fig. 5e). In the β-TCP group, mature collagen was found in the connective tissue between β-TCP granules, but almost no collagen was found in β-TCP granules (Fig. 5g). In the control group, immature collagen was found in host bone and new bone, and mature collagen was found in fibrous connective tissue (Fig. 5j).

Six-week postoperative groups

HE staining of β-TCP + autologous bone group replaced the transplanted autologous bone granules with almost completely new bone. However, about half of the transplanted β-TCP granules remained and were not completely replaced with new bone (Fig. 4b-1,2). In the autologous bone group, a large number of bone cells were observed, indicating that most of the transplanted autologous bone was absorbed and replaced with new bone (Fig. 4e-1,2). In the β-TCP group, replacement with new bone did not progress significantly compared to after 4 weeks, and more than half of the β-TCP granules remained (Fig. 4h-1,2). In the control group, there was a slight increase in new bone compared to 4 weeks, but the defect was dominated by fibrous connective tissue (Fig. 4k-1,2).

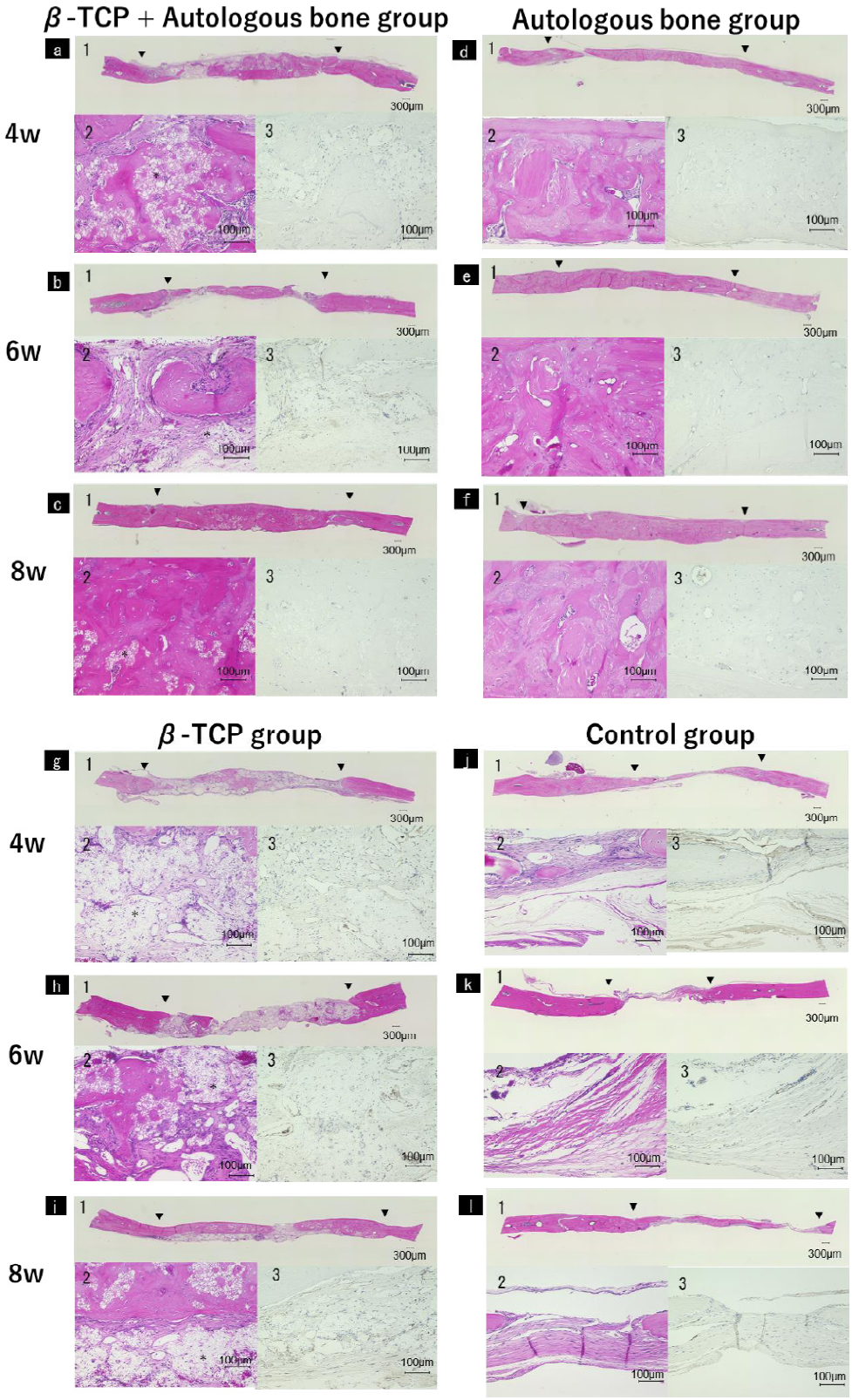

Fig. 4 Histological images of hematoxylin and eosin-stained and von Willebrand factor (vWF)-immunostained sections from each group at 4, 6, and 8 weeks after transplantation. Between▼: the defect; *: β-TCP granules. (a–c) β-TCP + autologous bone group; (d–f) Autologous bone group; (g–i) β-TCP groups; (j–l) control groups (1,2: hematoxylin and eosin-stain, 3: vWF immunostain). 1: original magnification, ×4; 2,3: original magnification, ×20.

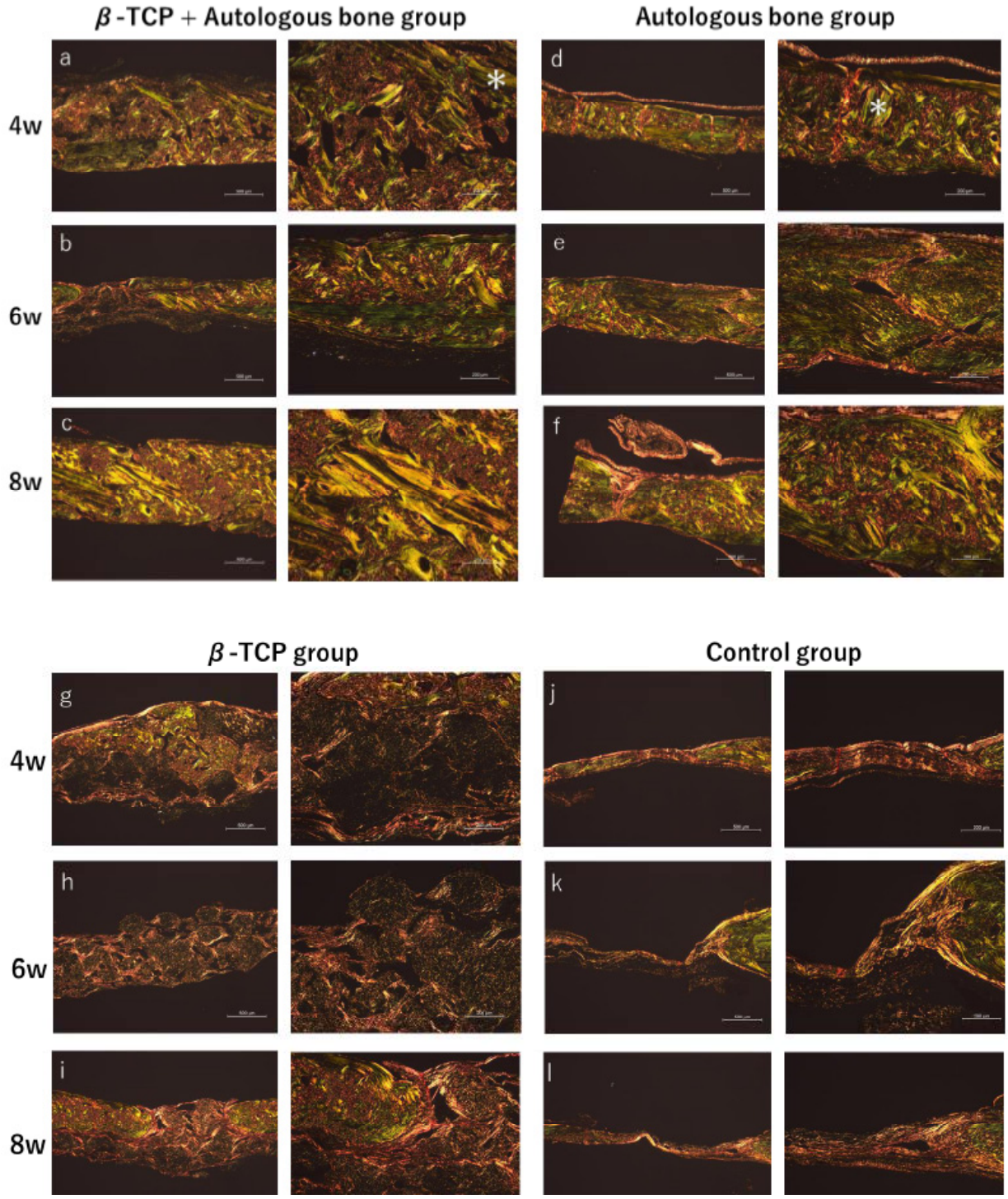

Fig. 5 Histological images observed with a polarizing microscope of picrosirius red staining in each experimental group 4, 6, and 8 weeks after transplantation. (a–c) β-TCP + autologous bones group; (d–f) Autologous bone group; (g–i) β-TCP group; (j–l) control group (red and yellow indicate mature collagen; green indicates immature collagen). Left, original magnification: ×4; Right, original magnification: ×10 (*: residual autologous bone granules). The bars are as follows: left, 500μm;right, 200μm.

In vWF immunostaining, almost no angiogenesis was observed in the autologous bone granules in the β-TCP + autologous bone group, but the β-TCP granules showed newly formed blood vessels, which was significantly different from the case of 4 weeks. There was no (Fig. 4b-3). A small number of vascular endothelial cells were found in the autologous bone group, but there was no significant change from 4 weeks (Fig. 4e-3). In the β-TCP group, there was no significant change from 4 weeks (Fig. 4h-3). In the control group, a small number of vascular endothelial cells were observed in the fibrous connective tissue at 4 weeks (Fig. 4k-3).

Under a polarizing microscope, picrosirius staining showed an increase in immature collagen between transplanted autologous bone granules and between β-TCP granules in the β-TCP + autologous bone group compared to 4 weeks, with an increase in mature collagen. It was observed in β-TCP granules (Fig. 5b). The autologous bone group showed an increase in immature collagen in new bone matrix coloring compared to that at 4 weeks, despite a decrease in mature collagen (Fig. 5e). In the β-TCP group, mature collagen derived from connective tissue was observed between β-TCP granules at 4 weeks, and a slight increase in immature collagen was observed in β-TCP granules (Fig. 5h). In the control group, immature collagen was observed in host bone and new bone, and mature collagen was found in fibrous connective tissue at 4 weeks (Fig. 5k).

Eight-week postoperative groups

HE staining and vWF immunostaining replaced autologous bone granules with new bone in the β-TCP + autologous bone group. In addition, although the transplanted β-TCP granules remained, they were slightly absorbed compared to 6 weeks, and a mixture of autologous bone-derived new bone and β-TCP granule-derived new bone was confirmed (Fig. 4c-1,2). Furthermore, it was shown that most of the transplanted autologous bones of the autologous bone group were replaced with new bones. However, the new bone became denser than the 6-week bone (Fig. 4f-1,2). In the β-TCP group, replacement with new bone was progressing compared with 6 weeks later. New bone was found more frequently laterally to the skull, and the remaining β-TCP granules were observed medially from the center of the defect (Fig. 4i-1,2). In the control group, the defect showed a slight increase in new bone compared to 6 weeks, but the new bone was thin and the defect was dominated by connective tissue (Fig. 4l-1,2).

vWF immunostaining showed a small number of vascular endothelial cells between autologous bone granules in the β-TCP + autologous bone group, but a large number of vascular endothelial cells were observed between β-TCP granules rather than between autologous bone granules. (Fig. 4c-3). A small number of vascular endothelial cells were found in the autologous bone group (Fig. 4f-3). In the β-TCP group, vascular endothelial cells were observed between the β-TCP granules, but few vascular endothelial cells were present in the new bone (Fig. 4i-3). In the control group, a small number of vascular endothelial cells were observed in the fibrous connective tissue (Fig. 4l-3).

Under a polarizing microscope, picrosirius staining showed an increase in mature and immature collagen in new bone and a decrease in immature collagen in the β-TCP + autologous bone group compared to 6 weeks. A mixture of immature collagen and mature collagen was found between the β-TCP granules, and an increase in mature collagen was also observed in the β-TCP granules (Fig. 5c). The autologous bone group showed an increase in mature collagen such as yellow and a decrease in immature collagen in the new bone matrix coloring (Fig. 5f). In the β-TCP group, an increase in immature collagen was observed in new bone. However, mature collagen was observed between the remaining β-TCP granules, and an increase in mature collagen was observed in the β-TCP granules compared to 6 weeks (Fig. 5i). In the control group, there was a slight increase in mature collagen in fibrous connective tissue compared to 6 weeks (Fig. 5l).

Discussion

Autologous bone is widely used in bone regenerative medicine as it contains growth factors [19]. However, autologous bone grafting has some drawbacks, such as invasiveness at the time of bone collection and the limited availability of collectable bone. However, due to the absence of growth factors, TCP has only osteoconduction ability and lacks osteoinduction ability. Currently, graft materials, such as autologous bone and TCP, are widely used as bone regeneration materials in clinical practice, but few studies have focused on bone quality to investigate the detailed mechanism of bone regenera-

tion.

We generated a skull defect model of male SD rats and transplanted β-TCP + autologous bone, autologous bone granules alone, or β-TCP granules alone at the bone defect. HE staining showed the fastest replacement with new bone in the autologous bone group among all transplant groups (Figure 4). In addition, picrosirius staining under a polarizing microscope showed that the proportion of immature collagen was initially high in the collagen in the new bone of the autologous bone group. However, the proportion of mature collagen increased over time, resulting in a mixture of immature and mature collagen at 8 weeks after transplantation (Figure 5d–f). In this study, the maturity of collagen fibers was evaluated by using yellow, orange, and red collagen fibers as mature collagen and green-like collagen fibers as immature collagen. Our finding was consistent with the report by Junqueira *et al.* [17] that type I collagen, which is a thick fiber, results in yellow, orange, and red polarized light, whereas type III collagen, which is a thin fiber, results in green-like polarized light in the picrosirius-polarization-method using a polarizing microscope [18]. Moreover, our finding was also consistent with the report by Glimcher *et al.* [20] showing that collagen matures as the maturity of regenerated bone increases. However, our micro-CT examination showed no increase in the BV/TV values from 4 to 6 weeks (Figure 3). This appears to contradict the proportional relationship between the amount of collagen and the BV/TV, in which the BV/TV value increases due to the addition of bone minerals (Ca^{2+}, HPO_4^{2-}) as collagen increases and matures. The lack of an increase in the BV/TV at 6 weeks in our study is likely because autologous bone granules remained at 4 weeks but not at 6 weeks, as shown through histological findings (Figure 5d,e). Since the bone mineral content of remaining autologous bone granules is considered to be higher than that of immature new bone, the lack of an increase in the BV/TV is likely the result of the decrease in remaining autologous bone granules due to resorption from 4 to 6 weeks. Importantly, this autologous bone granule transplantation procedure showed that the amount of remaining autologous bone granules affects the relationship between the BV/TV and the amount of collagen. In this study, the BV/TV increased from 6 to 8 weeks after transplantation. In contrast, Yasui *et al.* [21] demonstrated that the BV/TV decreased in the autologous bone group from 6 to 8 weeks after transplantation. Based on these results, because the replacement of transplanted autologous bone granules with new bone is delayed when the particle size of the transplanted autologous bone granules is larger than that used in our study, the BV/TV might decrease 8 weeks after transplantation due to the effect of remaining autologous bone granules from 6 to 8 weeks. Thus, the particle size of β-TCP in our study was set to 100–500 μm to promote bone formation and angiogenesis. However, an increase in the proportion of large particles of autologous bone granules also results in a greater effect on remaining autologous bone granules. Therefore, the particle size of the autologous bone granules to be transplanted should also be taken into consideration in BV/TV measurements.

Focusing on the state of the granules in the β-TCP group, β-TCP granules remained even 8 weeks after transplantation, and their replacement with new bone was minimal compared to that in the autologous bone and β-TCP + autologous bone groups (Fig. 5h–j). This was consistent with the slow replacement of β-TCP granules observed in a previous studyv [22]. In addition, HE staining, picrosirius red staining, and vWF immunostaining showed mature collagen derived from connective tissue and thick blood vessels in the space between granules at 4 weeks. However, little collagen was formed inside the granules, and only a small amount of immature collagen was observed. An increase in immature collagen derived from blood vessels and new bone was observed inside the granules over time, but mature collagen derived from new bone was barely seen. Morikawa demonstrated [23] that even small pores of β-TCP granules could be invaded by blood vessels and connective tissue, which was consistent with the result of our study. It is important to note that at each time-point, the formation and maturation of collagen inside the granules were shown to be faster in the β-TCP granules in contact with the periosteum on the parietal region than in those in contact with the dura mater (Figure 5g–i). This suggests that replacement with new bone is promoted by adjoining growth factors and cells even in materials with a porous structure that do not have growth factors like β-TCP.

Furthermore, we focused on the experimental results of the β-TCP + autologous bone group. It is widely known that autologous bone granules contain growth factors and cells. In the β-TCP + autologous bone group, invasion of collagen into β-TCP granules was evidently

faster compared to that in the β-TCP group at 4 weeks after transplantation (Fig. 5a). Furthermore, mature collagen derived from new bone in β-TCP granules and the replacement of β-TCP granules with new bone were observed 6 weeks after transplantation (Fig. 5b). At 8 weeks after transplantation, almost all β-TCP granules adjacent to the periosteum and autologous bone granules were replaced with new bone, and a large amount of mature collagen derived from new bone was found even inside β-TCP granules distant from the periosteum and autologous bone granules (Fig. 5c). This result indicates that adjoining autologous bone granules (growth factors) adjacent to β-TCP granules increased the rate and amount of collagen formation in β-TCP granules and promoted their replacement with regenerated bone.

It is highly important to note that our results revealed the mechanism underlying the collagen formation sequence during the formation of new bone. Following transplantation, immature collagen first emerges for the formation of new bone. As seen in the β-TCP group, a network of immature collagen was faintly observed in the β-TCP granules at 4 weeks (Fig. 5g). Then, after the increase in immature collagen in the β-TCP granules at 6 weeks, some immature collagen becomes mature collagen. Then, a mixture of mature and immature collagen is found in the newly formed bone at 8 weeks. A similar collagen status was observed not only in the β-TCP group but also in the host bone and the new bone of the β-TCP + autologous bone and the autologous bone groups at 8 weeks. Moreover, a study of rat marginal periodontitis reported that although most alveolar bone is composed of type I collagen (mature collagen), type III collagen (immature collagen) is present around the Havers canal and periosteum [24]. In addition, type III collagen is generally considered an immature form of type I collagen. Gay *et al.* [25] reported that in the wound healing process of connective tissue, type III collagen appears first, followed by the emergence of type I collagen, which is consistent with the findings of our study.

These results indicate that the β-TCP + autologous bone group exhibited the ability to form blood vessels into the graft material for a long period after transplantation compared to that in the autologous bone group, as well as the elevated production of collagen into β-TCP granules during the bone formation process compared to that in the β-TCP group. The bone regeneration material used in this study, which was a mixture of β-TCP granules and autologous bone granules, was shown to be highly superior because of not only its effect on space-making but also on the mature bone quality it acquires, which is comparable to that of existing bone and the autologous bone graft. Similar evaluations using a pre-clinical bone defect model with large animals are expected in the future.

Acknowledgement
This study was funded by JSPS Grant-in-Aid for Scientific Research No. JP17K17343.

Conflicts of Interest
All authors declare no conflicts of interest regarding the publication of this paper.

References
1) Hussain A, Takahashi K, Sonobe J, Tabata Y, Bessho K. Bone regeneration of rat calvarial defect by magnesium calcium phosphate gelatin scaffolds with or without bone morphogenetic protein-2. J Maxillofac Oral Surg 2014; 13: 29-35.
2) Sato N, Handa K, Venkataiah VS, Hasegawa T, Njuguna MM, Yahata Y, Saito M. Comparison of the vertical bone defect healing abilities of carbonate apatite, β-tricalcium phosphate, hydroxyapatite and bovine-derived heterogeneous bone. Dent Mater J 2020; 39: 309-318.
3) Anderson HC. Mechanism of mineral formation in bone. Lab Invest 1989; 60: 320-330.
4) Carreira A, Lojudice F, Halcsik E, Navarro R, Sogayar M, Granjeiro J. Bone morphogenetic proteins: facts, challenges, and future perspectives. J Dent Res 2014; 93: 335-345.
5) Dimitriou R, Jones E, McGonagle D, Giannoudis PV. Bone regeneration: current concepts and future directions. BMC Med 2011; 9: 1-10.
6) Wiedmann-Al-Ahmad M, Gutwald R, Gellrich N-C, Hübner U, Schmelzeisen R. Growth of human osteoblast-like cells on beta-tricalciumphosphate (TCP) membranes with different structures. J Mater Sci Mater Med 2007; 18: 551-563.
7) Carrodeguas RG, De Aza S. α-Tricalcium phosphate: Synthesis, properties and biomedical applications. Acta Biomater 2011; 7: 3536-3546.
8) Takabatake K, Yamachika E, Tsujigiwa H, Takeda Y, Kimura M, Takagi S, Nagatsuka H, Iida S. Effect of geometry and microstructure of honeycomb TCP scaffolds on bone regeneration. J Biomed Mater Res Part A 2014; 102: 2952-2960.

9) Osteoporosis prevention d, therapy. NIH Consensus development panel on osteoporosis prevention, diagnosis, and therapy. JAMA 2001; 285: 785-795.
10) Kuroshima S, Kaku M, Ishimoto T, Sasaki M, Nakano T, Sawase T. A paradigm shift for bone quality in dentistry: A literature review. J Prosthodont Res 2017; 61: 353-362.
11) Imbert L, Gourion-Arsiquaud S, Villarreal-Ramirez E, Spevak L, Taleb H, van der Meulen MCH, Mendelsohn R, Boskey AL. Dynamic structure and composition of bone investigated by nanoscale infrared spectroscopy. PLoS One 2018; 13: e0202833. doi: 10.1371/journal.pone.0202833.
12) Willett TL, Dapaah DY, Uppuganti S, Granke M, Nyman JS. Bone collagen network integrity and transverse fracture toughness of human cortical bone. Bone 2019; 120: 187-193.
13) Fernandes H, Mentink A, Bank R, Stoop R, Van Blitterswijk C, De Boer J. Endogenous collagen influences differentiation of human multipotent mesenchymal stromal cells. Tissue Engin Part A 2010; 16: 1693-1702.
14) Unal M, Creecy A, Nyman JS. The role of matrix composition in the mechanical behavior of bone. Curr Osteoporosis Rep 2018; 16: 205-215.
15) Ohnishi Y, Toda I, Suwa F. Measurement of interspaces after filling bone defects with different sizes of β-tricalcium phosphate granules: Comparison between experimental animals and defect models. J Osaka Dent Univ 2015; 49: 149-156.
16) Wu X-X, Gordon RE, Glanville RW, Kuo H-J, Uson RR, Rand JH. Morphological relationships of von Willebrand factor, type VI collagen, and fibrillin in human vascular subendothelium. American J Pathol 1996; 149: 283-291.
17) Junqueira LCU, Bignolas G, Brentani RR. Picrosirius staining plus polarization microscopy, a specific method for collagen detection in tissue sections. The Histochemical J 1979; 11: 447-455.
18) Lattouf R, Younes R, Lutomski D, Naaman N, Godeau G, Senni K, Changotade S. Picrosirius red staining: a useful tool to appraise collagen networks in normal and pathological tissues. J Histochem Cytochem 2014; 62: 751-758.
19) Weiland AJ, Phillips TW, Randolph MA. Bone grafts: a radiologic, histologic, and biomechanical model comparing autografts, allografts, and free vascularized bone grafts. Plast Reconstr Surg 1984; 74: 368-379.
20) Glimcher MJ. The nature of the mineral component of bone and the mechanism of calcification. Instr Course Lect 1987; 36: 49-69.
21) Yasui K, Tatumura M, Lyu J, Kurushima Y, Zhao M, Tsuda K, Deng W, Morikuni H, Nishiura A, Hashimoto Y. Evaluation of Bone Regeneration using Dentinal Granules with Autologous Bone. J Oral Tissue Engin 2020; 18: 13-22.
22) Ioku K. Hydroxyapatite and related calcium phosphates as ceramic biomaterials. Inorg Mater 1996; 3: 412-418.
23) Morikawa S. Compartive study on β-tricalcium phosphate and hydroxyapatite as bioactive artificial bone fillers. Tokyo Jikeikai Med J 2000; 115: 193-207.
24) Kamata M, Kamoi K. Histopathological study on qualitative changes in gingival collagen fibers for experimental periodontitis in rats. Remodeling of type I and III collagens detected by the Picrosirius-polarization method. JSP 1989; 31: 491-520.
25) Gay S, Vijanto J, Raekallio J, Penttinen R. Collagen types in early phases of wound healing in children. Acta Chir Scand 1978; 144: 205-211.

(Received: December 2, 2020/
Accepted: December 23, 2020)

Corresponding author*:*
Masayasu Tatsumura, D.D.S.
Department of Orthodontics,
Osaka Dental University
5-17,1-Chome, Otemae, Chuo-ku,
Osaka 540-0008, Japan
Tel: +81-80-5233-2690, +81-6-6910-1514
E-mail: masayasu-t@cc.osaka-dent.ac.jp